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Short communication

In vitro activity of *N*-chlorotaurine (NCT) in combination with NH_4Cl against *Trichomonas vaginalis*

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ABSTRACT

Trichomoniasis, caused by the protozoan *Trichomonas vaginalis*, is usually treated with metronidazole, however resistance is on the rise. In this study, *N*-chlorotaurine (NCT), a new endogenous mild active chlorine compound for topical use, killed *T. vaginalis* in vitro within 15 min of treatment at a concentration of 55 mM (1%), which is well tolerated by human tissue. The activity of NCT was further enhanced by addition of ammonium chloride (NH_4Cl). A combination of 5.5 mM (0.1%) NCT plus 19 mM (0.1%) NH_4Cl killed 100% of trichomonads within 5 min.

1. Introduction

Trichomonas vaginalis is the causative agent of trichomoniasis, the most common non-viral sexually transmitted disease worldwide. Typical symptoms are vaginitis and cervicitis in women; in men the infection can lead to chronic prostatitis syndrome [1]. Metronidazole is the drug of choice for trichomoniasis, however treatment during pregnancy is problematic [2] and resistance is increasing [3,4].

In a previous study, we demonstrated the amoebicidal effect of *N*-chlorotaurine (NCT) (Cl-HN-CH_2-CH_2-SO_3Na), thereby establishing the activity of this compound against protozoa [5]. NCT, the *N*-chloro derivative of the amino acid taurine produced by granulocytes and monocytes during the oxidative burst, is a long-lived oxidant that can be synthesised chemically [6] and applied topically for treatment of infections [7]. It has broad-spectrum microbicidal activity against bacteria, viruses, fungi and protozoa [5,7–9]. Phase I and II clinical studies have revealed high tolerability and therapeutic effects of regular applications of 55 mM (1%) NCT in the eye, sinuses, oral cavity, outer ear canal, skin ulcerations and urinary bladder [7,10].

The antimicrobial activity of NCT against bacteria [8], fungi [8,9] and free-living amoebae [5] can be enhanced markedly by addition of ammonium chloride (NH_4Cl) owing to the formation of monochloramine (NH_2Cl) in equilibrium by transfer of the active chlorine atom [6–9]. NH_2Cl is more lipophilic than NCT and penetrates microorganisms more rapidly. For special indications, e.g. treatment of viral conjunctivitis, addition of NH_4Cl to NCT might be an advantage [7].

The aim of the current study was to investigate the activity of NCT and its combination with NH_4Cl against *T. vaginalis* in vitro.

2. Material and methods

A metronidazole-susceptible (ATCC 30001) and a metronidazole-resistant (ATCC 50138) strain of *T. vaginalis* were investigated for their susceptibilities to NCT. Both strains were grown under microaerophilic conditions in trypticase–yeast extract–maltose (TYM) medium [11] at 37 °C and were subcultured every 72 h.

NCT was produced synthetically at Innsbruck Medical University (Innsbruck, Austria) and was used alone as well as in combination with NH_4Cl as previous studies had revealed a high synergistic potential for this substance [6,8]. NCT (molecular weight 181.57 g/mol) [6] was used at final concentrations of between 5.5 mM (0.1%) and 55 mM (1%) dissolved in either TYM or phosphate-buffered saline (PBS). Trichomonads were treated for 5, 15 and 60 min at 37 °C. Short intervals were chosen because stability testing revealed that the oxidative capacity of 55 mM NCT is reduced by 15% after 1 min, by 90% after 1 h and by >95% after 2 h in

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TYM. NH_4Cl was purchased from Sigma-Aldrich (Vienna, Austria) and was used in the molar ratio 1:3.4 and 1:29 (NCT:NH_4Cl) as in previous studies [5,8]. Control experiments revealed no antiparasitic effects of NH_4Cl alone. Additional experiments with 5.5 mM (0.1%) NCT plus 19 mM (0.1%) NH_4Cl (1:3.4) in PBS instead of TYM medium were performed to investigate the efficacy in the absence of chlorine consumption caused by unspecific reactions of NCT with the culture medium.

Viability of trichomonads was followed by phase contrast microscopy and trypan blue (0.4%) staining, and 50% and 90% effective concentrations (EC_{50} and EC_{90} values) were calculated using non-linear regression models. Cytotoxicity of NCT against vaginal epithelial cells (ATCC; CRL 2616) was investigated using the EZ4U test (Biomedica GmbH, Vienna, Austria), which is a tetrazolium reduction assay. All experiments were carried out in triplicate and were repeated in two independent set-ups. For each experiment, a control series without NCT and additives was included.

Single-factor analysis of variance (ANOVA) and Tukey's HSD test (SPSS 14.0; SPSS Inc., Chicago, IL) were used for statistical analyses. *P*-values of <0.05 were considered significant.

3. Results and discussion

NCT showed cytotoxic effects against both strains of *T. vaginalis* tested. Treatment with 55 mM NCT (1%), a concentration well tolerated by all human tissues tested so far [7], led to 100% killing of both strains tested within 15 min in TYM medium (Fig. 1). At lower concentrations in this medium, strain ATCC 30001 was more susceptible than strain ATCC 50138. The EC_{50} of NCT was 9.0 mM for strain ATCC 30001, whilst it was 28.7 mM for strain ATCC 50138 after 1 h of treatment. EC_{90} values were 9.8 mM and 36.7 mM, respectively.

The activity of NCT was markedly enhanced by the addition of NH_4Cl, explained by formation of the small and more lipophilic monochloramine (NH_2Cl), which penetrates pathogens better than NCT and leads to faster oxidation of intracellular proteins [6–8]. In strain ATCC 30001, only one-third of the concentration of NCT was needed to achieve 100% killing when NH_4Cl was added, i.e. 17 mM (0.3%) NCT plus 57 mM (0.3%) NH_4Cl (Fig. 1); moreover, the killing time was reduced to 5 min. This effect was even greater when experiments were carried out in PBS instead of culture medium. In PBS, co-treatment with 5.5 mM (0.1%) NCT plus 19 mM (0.1%) NH_4Cl also resulted in complete killing of trichomonads within 5 min (data not shown). Inactivation of NCT and the formed NH_2Cl by reaction with constituents of the TYM medium must be assumed to contribute significantly to delayed killing in this medium, particularly at concentrations below 10 mM.

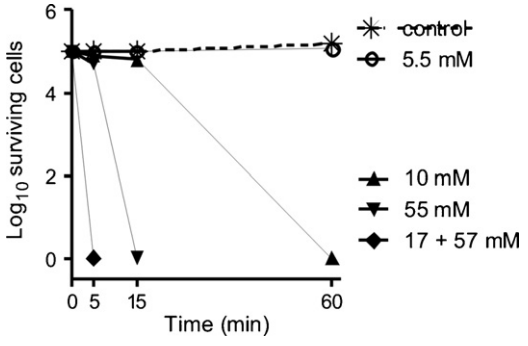

Fig. 1. Treatment of *Trichomonas vaginalis* ATCC 30001 with *N*-chlorotaurine (NCT) and co-treatment with NH_4Cl in trypticase–yeast extract–maltose (TYM) medium: ▼, 55 mM (1%) NCT; ▲, 10 mM (0.18%) NCT; ○, 5.5 mM (0.1%) NCT; ♦, 17 mM (0.3%) NCT + 57 mM (0.3%) NH_4Cl; --✳--, control. Mean value of three independent experiments; the standard deviation ranged between 0.14 and 0.21 $\log_{10}$ for values exceeding 4 $\log_{10}$ (not shown). $P < 0.01$ versus control for all zero survival values.

Such chlorine consumption in the presence of organic material, in particular reducing thio compounds, is well known [6,7]. It may also explain the relatively sharp threshold between highly active and inactive concentrations of NCT (Fig. 1). In strain ATCC 50138, higher concentrations of NH_4Cl (molar ratio 1:29) were necessary to achieve enhancement of activity in TYM medium.

The metronidazole-sensitive strain (ATCC 30001) was more susceptible to NCT than the metronidazole-resistant strain (ATCC 50138), and this difference was statistically significant ($P < 0.001$). An approximately three- to four-fold concentration of NCT required to kill 50% of the metronidazole-sensitive trichomonads was required to achieve the same killing rate in the metronidazole-resistant strain. Although the modes of action are different, since both metronidazole and NCT produce oxidative stress one could hypothesise that the more resistant strain has a better anti-oxidative protection system. This may be the case, but at the applied millimolar concentrations of NCT such mechanisms would not be sufficient. Since penetration of the pathogen rather than oxidation of the surface is the decisive step for killing by NCT [7], we assume that several factors, particularly differences in the membrane composition, may play a major role as has already been observed in other microorganisms [12].

In vivo studies have shown that 55 mM NCT (1%) is well tolerated by human mucosal tissue [7]. The in vitro tolerability of human cells in cell culture to NCT is known to be significantly (ca. 50–100-fold) lower [13–15]. However, vaginal epithelial cells were observed to be relatively resistant to NCT in the current study. The EC_{50} of NCT for these cells in vitro was 5.5 mM and the survival rate was 25% after treatment with 55 mM NCT for 1 h (Fig. 2). Tolerability to NCT was altogether significantly higher than to metronidazole, the standard drug for treatment of trichomoniasis. Increased tolerability of human cells to the agent in vivo compared with that in vitro can be explained by the fact that within tissues the cells are coherently organised in a structure affording better protection to oxidative agents. This has already been proven with human skin in vivo as well as human epidermis carcinoma cells in vitro [10].

The stability of the aqueous solution of NCT depends on temperature. At 37 °C its oxidation capacity decreases to zero within 3 weeks, whereas it remains at 75% after storage at room temperature for 2 months [6]. At sites of inflammation, oxidation capacity decreases more quickly owing to halogen-consuming proteins. After 15 min the oxidation capacity is reduced by 30–50% and after 2 h by up to 90% [8]. However, as trichomonads are killed within 15 min, and even faster with the addition of NH_4Cl, these decomposition rates are not likely to affect a possible therapeutic approach against trichomoniasis. Moreover, since human exudates demonstrated enhanced rather than decreased microbicidal activity of 0.5–1% NCT after topical application [8,9], it may be expected that NCT is also microbicidal against *T. vaginalis* in vivo after topical application.

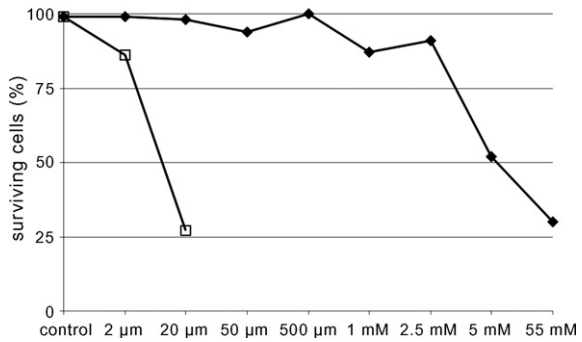

Fig. 2. Survival of vaginal epithelial cells treated for 1 h with *N*-chlorotaurine (NCT) (full diamond) or metronidazole (open square).

In conclusion, we have shown that NCT has strong trichomonacidal effects that are further enhanced by the addition of NH_4Cl. Further investigations of its utility in *T. vaginalis* infections are required.

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Competing interests: None declared.

Ethical approval: Not required.

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